IMPACT OF A VALIDATED, COST EFFECTIVE SCREEN FOR CIGUATERIC FISH

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Ciguatoxins contaminating ciguateric fish may be detected by a range of *in vivo* (e.g. mouse, cat, mosquito or chicken), *in vitro* (ELISA, atria) and chemical assays. Current research seeks a selective screen to detect low levels of ciguatoxin-1 (0.05–5.0ppb) in fish flesh or in an easy-to-prepare extract. This review summarises requirements for a validated, cost effective screen for ciguateric fish. Implementation of such a screen will reduce adverse health effects. An attendant benefit will be the improved marketability of reef fish.

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One important goal of present-day ciguatera research is the development of a cost-effective screen for the toxins contaminating ciguateric fish (Lewis,1993). The range of toxins involved in ciguatera has been the subject of some debate. It appears that only the ciguatoxins and analogues (e.g. gambiertoxins) are involved (Murata et al., 1990; Legrand et al.,1992; Lewis et al.,1991). These toxins are closely related in structure and all activate voltage-dependent sodium channels. Of these, CTX-1 typically contributes ~90% of the toxicity of ciguateric carnivorous fish in the Pacific (Legrand et al.,1992; Lewis & Sellin, 1992) and should be considered the primary target of a screen for ciguateric fish. Other ciguatoxin analogues and toxins such as okadaic acid, maitotoxins or *Trichodesmium* toxins are likely to play a minor role in human illness. However, these lower potency toxins could interfere with the response of a screen. The challenge for researchers is to develop a method that can rapidly and selectively screen CTX-1 which is present between 0.1 and 5ppb in the flesh of fish that cause ciguatera (Lewis,1992). Using a 2-fold risk factor to ensure public health is protected necessitates that the screen be capable of reliably detecting CTX-1 in fish flesh at 0.05ppb (50ppt) and above.

A number of assays have been used to detect ciguatoxin in fish. These include a range of *in vivo* assays (e.g. mouse, cat, chicken, mosquito: Banner et al.,1960; Kimura et al.,1982; Lewis & Endean,1984; Bagnis et al.,1985; Vernoux et al., 1985), a number of *in vitro* assays utilising antibodies (Hokama,1991) or isolated tissues (Kimura et al.,1982) and chemical assays involving derivatisation and HPLC separation with fluorescence detection (Legrand et al.,1992; Dickey et al.,1992). Biosensor-type assays are also under development and this approach holds much promise for the detection of ciguateric fish. These assays remain to be validated for their ability to selectively detect ciguatoxins in crude extracts of fish. We validated the mouse assay for ciguatoxin in up to 20mg of ether extract (Lewis & Sellin,1993). This assay was only able to detect CTX-1 at >0.5ppb in fish flesh. Importantly, this study found that 63± 14% of spiked ciguatoxin was recovered using a standard extraction procedure, thereby establishing its suitability for confirming whether a fish sample was toxic or not. The cost of such an assay, as well as its insufficient sensitivity and ethical considerations, preclude the use of the mouse assay for routine seafood monitoring programmes. This paper summarises requirements for a validated, cost-effective screen for ciguateric fish and discusses some of the impacts of such a test on fisheries and fisheries products.

FEATURES OF A USEFUL SCREEN

A useful screen for detecting ciguateric fish needs to possess the following features: 1, simple implementation; 2, ready availability and long shelf life; 3, toxin selectivity proportional to the human oral potency of the ciguatoxin analogues (ie highest affinity for CTX-1); 4, it should yield a linear response over the range of toxin concentrations encountered in ciguateric fish; 5, acceptable accuracy (±20%) at the level of 0.05ppb CTX-1 that is independent of the fish tested; 6, high recovery of CTX-1 (>60%) during extraction and clean-up (>30% in exceptional circumstances); and 7, total cost of screen must be acceptable to the consumer.

Prototype stick tests (Hokama,1985; Hokama et al.,1985; Hokama,1991) have many of the

above features, but this or modified versions of the test are still not readily available. Published results of screening of fish suggest (but do not prove) that these tests have sufficient sensitivity (i.e. few false positive results reported). However, the apparent high frequency of false positive results (i.e. non-toxic fish rejected) suggests that the antibody employed may have a relatively low specificity for CTX-1.

A screen that utilises high affinity binding of ciguatoxin to a cheap protein substrate (e.g. IgG) and couples this interaction to a simply measured response (e.g. a colour change) has a high likelihood of achieving an adequate compromise between accuracy and cost. Such antibody-based screens have the potential to detect levels of analyte as low as 10^{-12}M in food (Gazzaz et al., 1992). However, matrix effects associated with the type of sample screened can often dramatically reduce assay sensitivity. The extent of such matrix effects can also vary with the solubility characteristics of the analyte. For solid food matrices, enzyme linked immunosorbent assays (ELISA) detected okadaic acid in shellfish of 10-300ppb (DSP-check, Ube Industries, Ltd, Tokyo) and detected aflatoxins above 10ppb (Dorner & Cole,1989; Trucksess et al.,1989) or more recently above 1 ppb in a range of solid foods (Agri-Screen test for aflatoxins, Neogen Corporation, Michigan). The challenge is to develop a rapid screen for ciguatoxins that has one to two orders of magnitude greater sensitivity than presently available ELISA assays. A variety of approaches may be used to improve the sensitivity of antibody-based assays: (i) production of higher affinity antibodies (ii) optimising assay conditions in which the antibody is used (iii) amplifying the assay signal (not always accompanied by improved signal to noise) (iv) optimising sample extraction and clean-up (can add a significant cost). The potential of the latter approach is indicated for ciguatoxin which can be concentrated from the levels in flesh of 0.1-5ppb to levels of ~30–5,000ppb in a crude lipid extract with a two-step clean-up procedure that has 63% efficiency (Lewis & Sellin,1993).

Toxins responsible for ciguatera arise through the biotransformation of a number of gambiertoxins produced by *Gambierdiscus toxicus* (Murata et al.,1990; Holmes et al.,1991; Legrand et al.,1992). So a range of low potency forms of the ciguatoxins and gambiertoxins (including CTX-2 and CTX-3) could accumulate in fish. These low potency forms may be detected with antibodies raised to CTX-1 and if so they could give rise to false positive results. To-date the cross-reactivity of the antibodies in use for the various ciguatoxin analogues has not been established.

An acceptable cost for ciguatera screening has not been determined. This will relate to the added value screened fish will attract in the marketplace. An add-on cost of less than 10% of the value of the product may be reasonable. The 'cost' of a screening programme should incorporate an estimate of the cost of discarding non-toxic fish as a result of false positive results. It may be possible to reduce the cost of screening by pooling fish samples prior to screening. Such an approach requires a highly sensitive screen but could work where ciguatera is a low level problem (e.g. Australia). This approach could not work where non-toxic fish have levels of ciguatoxin within an order of magnitude of levels that cause human poisoning.

A blind screening of ciguateric fish from Australia (specimens confirmed ciguateric by human and mice assays) with a prototype of the Ciguatect™ test kit (November,1991) revealed that there were few, if any, false positive results. However, there was a strong possibility that some ciguatoxic samples went undetected by the test. In fact five of six of the ciguateric fish samples are likely to have given a false negative result. Further testing of these fish using later modifications of the Ciguatect™ test are not reported and no satisfactory explanation has been given for the conflicting results obtained. These results were obtained at a time when the test was being considered for commercial release. Since this time modifications to the test have been made but a final format for the Ciguatect™ test remains elusive.

TYPES OF ANTIBODY ASSAYS AVAILABLE

Several approaches are available for incorporating antibodies (or similar proteins) into an assay format for detecting haptens such as ciguatoxin (Fig.1). In all cases a label, be it a radioisotope, an enzyme or a luminescent or fluorescent probe, is used to detect the targeted compound. Each approach has strengths and weaknesses but all require a high affinity antibody that is selective and specific for the targeted hapten. The first assay considered is the indirect hapten assay which requires that the targeted hapten (e.g. ciguatoxin) is first non-selectively immobilised to a solid support (along with

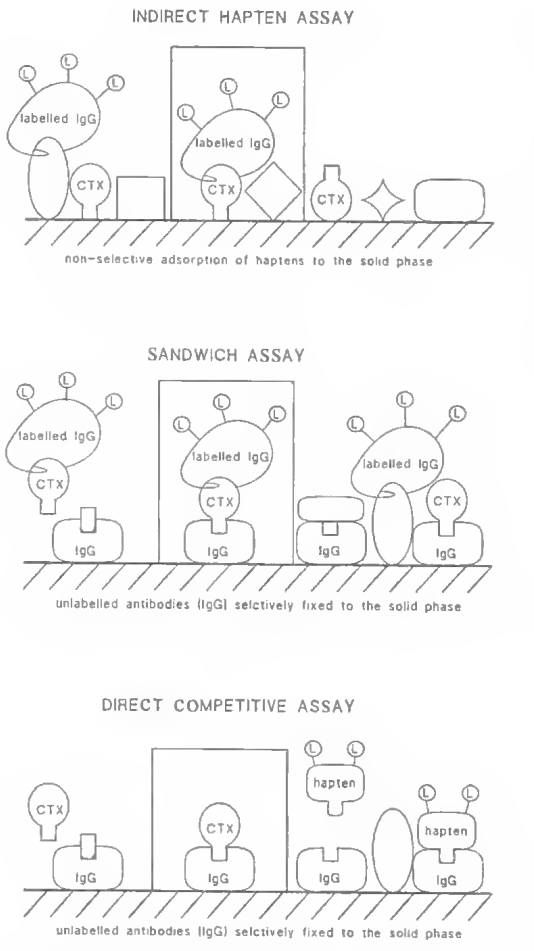

FIG.1. Antibody assays available for the detection of haptens such as ciguatoxin (CTX). Either the IgG or hapten are labelled (L) with probe to indicate the presence of the targeted hapten. Other shapes represent the range of compounds present along with the targeted hapten. The IgG could be replaced with other proteins possessing a high affinity for the targeted hapten (eg the sodium channel could be used for detecting ciguatoxin). The large box in each diagram surrounds the interaction responding to the presence of targeted hapten, while interactions beyond this box result in a reduced (false negative) or enhanced (false positive) assay response that is unrelated to the presence of the targeted hapten.

numerous other contaminants) prior to its detection with a labelled antibody specific for the part of the hapten left exposed following binding. In practice, it is often not possible to obtain an antibody that can still 'see' small haptens bound to a solid phase, but this approach can allow rapid detection of a hapten without a time-consuming extraction step. Tests developed for ciguatoxins by Hokama (1991) and more recently by Hawaii Chemtect (Pasadena) have used this approach in the development of a screen for ciguateric fish.

The second approach is to develop a sandwich assay. This requires development of two antibodies which can mutually bind the targeted hapten. This assay can be more selective than the direct assay although binding reactions in two-site immunoassays are complex and not easily predicted (Boscato et al.,1989). This assay is limited by the difficulties associated with obtaining two antibodies which don't interfere with each others binding to a small hapten such as ciguatoxin.

The third approach is the direct competitive assay which requires that a second hapten is available that competes with the targeted hapten for binding to an antibody. With this approach either the hapten or the antibody can be fixed to the solid phase. Unlike the first two assays, the response is inversely proportional to the targeted hapten, but such a response is likely to be more specific for the targeted hapten than the former assays. The direct competitive assay likely holds most promise for an accurate screen but requires a competing hapten to be found. While it is possible that the sandwich and competitive assays could be developed for the direct sampling of fish flesh, it is likely is that extraction and clean-up steps will be required. The solubility requirements of the hapten and stability of antibody in solvent need to be considered if the assay is designed to detect solubilised hapten.

SCREEN VALIDATION

A number of criteria need to be met before a screen for ciguateric fish can be considered useful, including: 1, the screen must detect all fish samples confirmed ciguateric following human consumption; 2, the screen must have an acceptably low rate of false positives (i.e. a false positive rate within an order of magnitude of the reported ciguatera incidence for the species tested) and the cost of false positive results must be included in the cost of screening; 3, the screen must detect spiked CTX-1 in crude fish extracts at levels occurring naturally and should detect CTX-1 spiked in a fish flesh homogenate; 4, the screen should produce appropriately accurate results for toxic and non-toxic fish both within and between laboratories. Wherever appropriate, negative controls should be run in a pair-wise design and results for these should be negative.

Absence of readily available reference toxins of the ciguatoxin class and lack of a validated analytical method to quantify the level and types of toxins present in samples of fish hinder attempts to validate screening tests for ciguateric fish. The use of the mouse bioassay to assess ciguatoxin levels (Lewis & Sellin in press) is presently the best available alternative to an *in vitro* approach. It could prove misleading to use other *in vivo* bioassays to validate screening tests at this time, especially the unreliable brine shrimp assay (R.J. Lewis unpubl. data) which has recently been used by D.L. Park to characterise the toxins present in fish screened by the S-PIA version of the Ciguatect™ test (Hungerford,1993).

To ensure reliability of a screen, any limitations of a screen with regard to species, sample preparation and storage etc should be well documented. Ideally, the screen should 'work' for all potentially ciguateric fish, irrespective of how they are caught or handled prior to sale. Accuracy must be regularly evaluated with reference to toxic and non-toxic specimens to ensure reliability over time. A sound basis needs to be established for any variation in methodology between control and test procedures.

IMPACT OF A SCREEN FOR CIGUATERIC FISH

Implementation of a useful screen will result in improved marketability of seafood captured in ciguatera endemic areas. Removal of toxic fish before consumption will lead to improved community health standards. With the availability of a screen come possibilities for opening fisheries for species which are presently restricted because of ciguatera. In Queensland new and potentially lucrative fisheries for red bass and perhaps chinaman fish and paddletail could be established once an effective screen is available.

Screening for ciguatera could be conducted at a number a levels in the chain of marketing of fish that includes fisherpersons, wholesalers, commercial companies, government agencies or consumer. Problems are likely to exist for the consumer seeking compensation to prove that the test was indeed performed on the fish involved in the poisoning according to the manufacturers instructions. It may even be necessary to show that the toxin involved was indeed a ciguatoxin. Who ends up conducting the test will depend on the final format of the test, government requirements and the level of risk of ciguatera associated with the fish being screened. Cost of screening will increase if more than one point of testing is required. It is interesting to speculate on the fate of a screening product that is shown to have failed to detect a toxic fish, especially if the fish results in a poisoning episode.

Another issue to be considered is what fish are to be screened. In some areas it might be appropriate to screen all potentially ciguateric reef fish, whereas in other areas only the high risk species presently marketed may need to be screened. Other classes of fish that may require a differential approach include (i) fish presently banned as a result of the threat of ciguatera they pose (ii) fish destined for export and (iii) large whole fish in one of the above categories.

LITERATURE CITED

BAGNIS, R., CHANTEAU, S., CHUNGUE, E., DROLLET, J.H., LECHAT, I., LEGRAND, A.M., POMPON, A., PRIEUR, C., ROUX, J. & TETARIA, C. 1985. Comparison of the cat bioassay, the mouse bioassay and the mosquito bioassay to detect ciguatoxicity in fish. Pp. 491–496. In Gabrie, C. & Salvat, B. (eds), 'Proceedings of the Fifth International Coral Reef Congress, Tahiti, Vol 4'. (Antenne Museum-Ephe: Moorea).

BANNER, A.H., SCHEUER, P.J., SASAKI, S., HELFRICH, P. & ALENDER, C.B. 1960. Observations on ciguatera-type toxin in fish. Annals New York Academy of Science 90: 770–787.

BOSCATO, L.M., EGAN, G.M. & STUART, M.C. 1989. Specificity of two-site immunoassays. Journal of Immunological Methods 117: 221–229.

DICKEY, R.W., BENCSATH, F.A., GRANADE, H.R. & LEWIS, R.J. 1992. Liquid chromatographic-mass spectrometric methods for the determination of marine polyether toxins. Bulletin de la Société de Pathologie Exotique 85: 514–515.

DORNER, J.W. & COLE, R.J. 1989. Comparison of two ELISA screening tests with liquid chromatography for determination of aflatoxins in raw peanuts. Journal Association Official Analytical Chemists 72: 962–964.

GAZZAZ, S.S., RASCO, B.A. & DONG, F.M. 1992. Application of immunochemical assays to food analysis. Critical Reviews in Food Science and Nutrition 32: 197–229.

HOKAMA, Y. 1985. A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23: 939–946.

HOKAMA, Y. 1991. Immunological analysis of low molecular weight marine toxins. Journal Toxicology - Toxin Reviews 10: 1–35.

HOKAMA, Y., OSUGI, A.M., HONDA, S.A.A., MATSUO, M.K. 1985. Monoclonal antibodies in the detection of ciguatoxin and other toxic polyethers in fish tissues by a rapid poke stick test.

Pp. 449–455. In Gabrie, C. & Salvat, B., (eds), 'Proceedings of the Fifth International Coral Reef Congress, Tahiti, Vol 4'. (Antenne Museum-Ephe: Moorea).

HOLMES, M.J., LEWIS, R. J., POLI, M.A. & GILLESPIE, N.C. 1991. Strain dependent production of ciguatoxin precursors (gambiertoxins) by *Gambierdiscus toxicus* (Dynophyceae) in culture. Toxicon 29: 761–775.

HUNGERFORD, J.M. 1993. Seafood toxins and seafood products. Journal AOAC International 76: 120–130.

KIMURA, L.H., HOKAMA, Y., ABAD, M.A., OYAMA, M. & MIYAHARA, J.T. 1982. Comparison of three different assays for the assessment of ciguatoxin in fish tissues: radioimmunoassay, mouse bioassay and *in vitro* guinea pig atrium assay. Toxicon 20: 907–912.

LEGRAND, A-M., FUKUI, M., CRUCHET, P., ISHIBASHI, Y. & YASUMOTO, T. 1992. Characterization of ciguatoxins from different fish species and wild *Gambierdiscus toxicus*. Pp. 25–32. In Tosteson, T.R., (ed.), ' Proceedings Third International Conference on Ciguatera Fish Poisoning, Puerto Rico'. (Polyscience Publications: Québec).

LEWIS, R.J. 1992. Ciguatoxins are potent ichthyotoxins. Toxicon 30: 207–211.

LEWIS, R.J. 1992. Socioeconomic impacts and management of ciguatera in the Pacific. Bulletin de la Société de Pathologie Exotique 85: 427–434.

LEWIS, R.J. & ENDEAN, R. 1984. Ciguatoxins from the flesh and viscera of the barracuda, *Sphyraena jello*. Toxicon 22: 805–810.

LEWIS, R.J. & SELLIN, M. 1992. Multiple ciguatoxins in the flesh of fishes. Toxicon 30: 915–919.

LEWIS, R.J. & SELLIN, M. in press. Recovery of ciguatoxin from fish flesh. Toxicon.

LEWIS, R.J., SELLIN, M., POLI, M.A., NORTON, R.S., MACLEOD, J.K. & SHEIL, M.M. 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29: 1115–1127.

MURATA, M., LEGRAND, A.M., ISHIBASHI, Y., FUKUI, M. & YASUMOTO, T. 1990. Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. Journal of the American Chemical Society 112: 4380–4386.

TRUCKNESS, M.W., STACK, M.E., NESHEIM, S., PARK, D.L., & POHLAND, A.E. 1989. Enzyme-linked immunosorbent assay of aflatoxins B_1, B_2, and G_1 in corn, cottonseed, peanuts, peanut butter, and poultry feed: a collaborative study. Journal o f the Association Official Analytical Chemists 72: 957–962.

VERNOUX, J.P., LAHLOU, N., MAGRAS, L.Ph. & GREAUX, J.B. 1985. Chick feeding test: a simple system to detect ciguatoxin. Acta Tropica 42: 235–240.